IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of: **Jon A. Wolff, Vladimir G. Budker**

Serial No.: **09/707,117**

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Group Art Unit: **1632**

Examiner: **Michael C. Wilson**

For: **Intravascular Delivery of Nucleic Acid**

DECLARATION UNDER 37 C.F.R. §1.132

Assistant Commissioner for Patents
Washington, DC 20231

Dear Sir:

I, Jon A. Wolff, hereby declare as follows:

1. I am an inventor of the captioned application.

2. I submit with this Declaration and Response further experimental material (attached) illustrating *in vivo* delivery of nucleic acid-containing viral and non-viral vectors and unexpressed functional nucleic acid to nonvascular muscle cells. This material demonstrates a correlation between delivery of naked DNA, plasmid-containing non-viral complexes, viral complexes and functional non-expressed nucleic acid (siRNA). No new matter was used in the experiments.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

5/9/03

Jon A. Wolff Date

[SUPPLEMENTAL INFORMATION]

A. Delivery to Rat Skeletal Muscle cells In Vivo Using Intra-iliac Injection.
Delivery of negatively charged PEI/DNA and histone H1/DNA complexes to skeletal muscles in rat via a single injection into the iliac artery: PEI/DNA and histone H1/DNA particles were injected into rat leg muscle by a single intra-arterial injection into the external iliac. Female Harlan Sprague Dawley rats, approximately 150 g, each received complexes containing 100 μg plasmid DNA encoding the luciferase gene under control of the CMV enhancer/promoter (pCI-Luc) [Zhang et al. 1997]. Rat iliac injections of 10 mL of solution (n = 2) were conducted as previously described (see example 8 of patent application 09/707,117). Results of the rat injections are provided in relative light units (RLUs) and micrograms (μg) of luciferase produced. To determine RLUs, 10 μl of cell lysate were assayed luminometer and total muscle RLUs were determined by multiplying by the appropriate dilution factor. To determine the total amount of luciferase expressed per muscle we used a conversion equation that was determined in an earlier study [Zhang et al. 1997] [pg luciferase = RLUs x 5.1 x 10^{-5}].

Table 17. Luciferase expression in multiple muscles of the leg following injection of negatively charged DNA/PEI or DNA/Histone HI particles.

DNA/PEI particles (1 : 0.5 charge ratio)

Muscle Group	*Total RLUs*	*Total Luciferase*
muscle group 1 (upper leg anterior)	3.50×10^9	0.180 μg
muscle group 2 (upper leg posterior)	3.96×10^9	0.202 μg
muscle group 3 (upper leg medial)	7.20×10^9	0.368 μg
muscle group 4 (lower leg posterior)	9.90×10^9	0.505 μg
muscle group 5 (lower leg anterior)	9.47×10^8	0.048 μg
muscle group 6 (foot)	6.72×10^6	0.0003 μg

DNA/histone H1 particles (1 : 0.5 charge ratio)

Muscle Group	*Total RLUs*	*Total Luciferase*
muscle group 1 (upper leg anterior)	3.12×10^9	0.180 μg
muscle group 2 (upper leg posterior)	9.13×10^9	0.202 μg
muscle group 3 (upper leg medial)	1.23×10^{10}	0.368 μg
muscle group 4 (lower leg posterior)	5.73×10^9	0.505 μg
muscle group 5 (lower leg anterior)	4.81×10^8	0.048 μg
muscle group 6 (foot)	6.49×10^6	0.0003 μg

Results indicated delivery of the negatively charged complexes containing luciferase-expressing plasmid to muscles throughout the leg via injection into a afferent artery.

B. Adenoviral vectors can be delivered to muscle parenchymal cells by an intravascular route. An adenoviral vector CMVLacZ that expresses the E. coli β-galactosidase from the immediate early promoter of the human cytomegalovirus (CMV) was prepared as previously described (Yang et al. 1996) The rat iliac artery injection was performed as above. 0.5 mg of papaverine and 40 ng of collagenase in 3 ml saline was pre-injected while blocking the iliac artery and vein. 5×10^8 particles of the adenoviral vector CMVLacZ in 10 ml of saline was

injected in about 10 sec. After 2 minutes, the clamps were opened. Two days after injection, leg muscle cells were assayed for luciferase as above. Delivery was monitored be expression of luciferase encoded within the adenovirus genome. The results summarized in Table 24 demonstrate the delivery of Adenovirus to multiple muscle groups in the leg.

Table 24: Delivery of adenovirus expressing a luciferase gene to skeletal muscle via iliac injection.

Muscle Group	Luciferase (ng)
Upper Leg Anterior	59.04
Upper Leg Posterior	18.33
Upper Leg Medial	4.44
Lower Leg Posterior	11.04
Lower Leg Anterior	5.33
Foot	0.22
Total	98.40

C. Delivery of siRNA to muscle cells in rat via an intra-iliac administration route: 10 μg pGL3 control and 1 μg pRL-SV40 with 5.0 μg siRNA-luc+ or 5.0 siRNA-ori were injected into iliac artery of rats. Specifically, animals were anesthetized and the surgical field shaved and prepped with an antiseptic. The animals were placed on a heating pad to prevent loss of body heat during the surgical procedure. A midline abdominal incision was made after which skin flaps were folded away and held with clamps to expose the target area. A moist gauze was applied to prevent excessive drying of internal organs. Intestines were moved to visualize the iliac veins and arteries. Microvessel clips were placed on the external iliac, caudal epigastric, internal iliac, deferent duct, and gluteal arteries and veins to block both outflow and inflow of the blood to the leg. An efflux enhancer solution (e.g., 0.5 mg papaverine in 3 ml saline) was injected into the external iliac artery though a 25 g needle, followed by the plasmid DNA and siRNA containing solution (in 10 ml saline) 1-10 minutes later. The solution was injected in approximately 10 seconds. The microvessel clips were removed 2 minutes after the injection and bleeding was controlled with pressure and gel foam. The abdominal muscles and skin were closed with 4-0 dexon suture.

Four days after injection, rats were sacrificed and the quadriceps and gastrocnemius muscles were harvested and homogenized. Luc+ and Renilla Luc activities were assayed using the Dual Luciferase Reporter Assay System (Promega). Ratios of Luc+ to Renilla Luc were normalized to the siRNA-ori control. siRNA-Luc I inhibited Luc+ expression in quadriceps and gastrocnemius by 85% and 92%, respectively, compared to the control siRNA-ori. Thus siRNA was effectively delivered to muscle cells in the leg using the delivery procedure.